

Original Research Article

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Morphological and Molecular Characterization of *Magnaporthe grisea* and Bio-Efficacy of *Bacillus* Strains against *M. grisea*

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ABSTRACT

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A study was undertaken to isolate, identify and characterize the rice blast pathogen *M. grisea* from different rice growing regions. In the present study, ten isolates of *M. grisea* were categorized into different groups based on colony colour and texture. PCR was performed to identify the *M. grisea* isolates using the universal primers of 18S (ITS 1) and 28S (ITS 4). The PCR reaction allowed amplifying the fungal ITS fragments of 550 bp. All the isolates had the expected specific size of 550 bp which depicts molecular based confirmation of *M. grisea*. Biocontrol approaches help to develop an eco-friendly sustainable management strategy for controlling the diseases. In this connection, totally ten strains of *Bacillus* spp. were screened against six isolates of *M. grisea* to test their efficacy. Among the strains, BS5 showed maximum mycelial growth inhibition followed by BS26.

Introduction

Rice is a major staple food and is consumed by nearly half of world's population. Rice is grown in warm or cool humid subtropic areas. The humid tropical environments in Asia are highly conducive for the rice disease epidemics. Rice is infected by various plant pathogens. Among them blast diseases is a most devastating disease (Susan *et al.*, 2018). The blast pathogen infects the crop in all stages of its growth, starting from nursery to grain falling stage under favorable

environmental condition. The disease causes heavy yield losses ranging from 35-50% during the epidemic years (Zhu *et al.*, 2005; Dean *et al.*, 2012). The disease is mainly noticeable when the pathogen attacks the leaf collar, nodes, leaf blades, necks & panicles. The lesions or spokes first appear as minutes brown speakes and eventually grows became spindle shaped. The center is grayish with brown margin. The lesions may expands and ultimately coalesce thus killings the entire leaf. The development of rice blast is favoured by a number of factors such as

high relative humidity (above 80%), low temperature (15°C-26°C), cloudy weather, more number of wet or rainy days, extended dew durations, slow wind movement, availability of collateral hosts and excessive doses of nitrogenous fertilizers. Morphological characterization has the overlapping of characters used for species classification frequently that makes difficult for identification and time intensive. Correct identification is necessary in order to adopt effective agricultural measures as soon as possible.

Therefore alternative approaches must be developed to accurately identify and differentiate fungal species. Recently many molecular approaches including Polymerase Chain Reaction (PCR) have been tested to identify plant pathogenic fungi (Langrell *et al.*, 2011). In this study the PCR technique was used to identify the *Magna porthegrisea*.

Many effective pesticides have been recommended against this pest and disease, but not considered as a long-term solution because of concerns about pesticide residue risks, health and environmental hazards, expense, residue persistence, pest resurgence and elimination of natural enemies. Biocontrol approaches help to develop an ecofriendly sustainable management strategy for controlling the diseases. The role of Plant Growth Promoting Rhizobacteria (PGPR) *viz.*, *Pseudomonas fluorescens* and *Bacillus subtilis* in biocontrol approaches for managing the pathogen in crop plants are well reported (Prabhukarthikeyan *et al.*, 2014a; 2017a, b; 2018). Among PGPR, the sporulating Gram-positive bacteria like *Bacillus* spp. have also been used successfully for plant disease control (Klopper *et al.*, 2004). The aim of the study is to isolate, identify the pathogen and testing the bio-efficacy of bacillus strains against *M. grisea* under *in vitro* conditions.

Materials and Methods

Isolation of Pathogen

The isolation of *M. oryzae* from blast infected samples was carried out under aseptic conditions. Infected leaves were brought to laboratory and washed thoroughly under running tap water and blot dries the leaves on filter paper. Infected tissues were cut into small pieces of 1 to 1.5 cm, surface sterilized with 70% ethanol for 45-60 second and washed in 0.1% HgCl₂ for 1-2 minutes. Give three washings to that tissue in sterilized distilled water. Then washed thrice with sterile distilled water and then placed in Oat Meal Agar (OMA) medium. Then wrap it with parafilm and kept upside (normally) upper side up and kept at 25±1 °C for fungus to grow. After 3-4 days transfer the fungus to the slants and processed for pure culture.

Morphological characterization

The *M. oryzae* isolates were further sub-cultured on oat meal agar medium and the morphological characters such as colony colour, colour of the mycelium, and surface appearance were studied.

Molecular characterization

DNA extraction

The culture of *M. grisea* isolates maintained in OMA slants were transferred to OMA plate and incubated at 28 °C for 2 to 4 days. Then transferred into 250 ml Erlenmeyer flask containing 150 ml OMA broth and was incubated at room temperature for 7 days. Mycelium was harvested by filtration through sterile filter and stored at -80 °C until used for DNA extraction. To extract the DNA, 1 g of frozen mycelium was ground to fine powder in liquid nitrogen and incubated in 5 ml, 2 per cent CTAB extraction buffer (10 mM trisbase

(pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, CTAB (2%), mercaptoethanol (0.1%) and PVP (0.2%) at 65^oC for 1 h. The suspension was added with equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) mixture. It was vortexed to mix two phases, followed by a centrifuge at 12, 000 rpm for 5 min. The supernatant was transferred to clean tube and mixed with equal volume of ice cold isopropanol. It was incubated at 25 ^oC for DNA precipitation. The precipitate was collected by centrifugation and the pellet was washed with 0.1 M ammonium acetate in 70 per cent ethanol. Again incubation was given for 15 min. The pellet was re-suspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and the DNA concentration was estimated spectrophotometrically.

PCR amplification of ITS region

Internal transcribed spacer regions in fungi have been found to evolve faster and therefore may contain sufficient nucleotide sequence variation to infer relationships between species. The reaction mix for PCR amplification of the DNA consisted of 20 µl vol, (0.25 mM each of primer pair, 0.25 mM dNTP, 1.5 mM MgCl₂, 50-80 ng of template DNA, 2 U of Taq DNA polymerase and 1x PCR buffer mix) and the sequence of the ITS primers were as follows:

Forward primer (ITS1):
TCCGTAGGTGAACCTGCGG

Reverse primer (ITS4):
TCCTCCGCTTATTGATATGC

PCR was undertaken using a Master cycler programmed for initial denaturation at 94^oC for 5 min, followed by 35 cycles consisting of denaturing at 94 ^oC for 1 min, 58 ^oC annealing for 1 min, extension at 72 ^oC for 1 min and with a final extension at 72 ^oC for 10 min (White *et al.*, 1990). The PCR

products were resolved on a 1.2% agarose at 80 voltage, stained (ethidium bromide 0.5 µg ml⁻¹), photographed and analyzed using Alpha Innotech gel documentation system.

Screening of antagonistic bacteria under *in vitro* conditions

The antifungal efficacy of *Bacillus* strains were tested by dual culture technique (Dennis and Webster, 1971) using PDA/OMA medium. A mycelial disc of the pathogen (9mm dia.) *M. grisea* was placed at one end of the plate and the bacterial antagonists were streaked at the periphery of the Petri dish just opposite to the mycelial disc of the pathogen. The plates were incubated at 26±2^oC.

The mycelial growth of the pathogen and inhibition zone was measured after 7 days incubation. *Bacillus* strains were obtained from Plant Pathology, ICAR-National Rice Research Institute, Cuttack and used for all the study. BS1 (MH257581), BS6 (MH251943), BS16 (MH257584) and BS26 (MH257586) were *B. subtilis*; two strains BS10 (MH257582) and BS39 (MH251913) as *B. cereus*; while, strains BS5 (MH251872), BS11 (MH257583), BS17 (MH257585) and BS32 (MH257588) were *B. amyloliquefaciens*, *B. megaterium*, *B. luciferensis* and *B. toyonensis*, respectively.

Statistical analysis

The data were statistically analyzed using the IRRISTAT version 92 developed by the International Rice Research Institute Biometrics unit, the Philippines (Gomez and Gomez, 1984). Prior to statistical analysis of variance (ANOVA) the percentage values of the disease index were arcsine transformed. Data were subjected to analysis of variance (ANOVA) at two significant levels ($P < 0.05$ and $P < 0.01$) and means were compared by Duncan's Multiple Range Test (DMRT).

Results and Discussion

Isolation from the infected leaf

The pathogen was isolated from infected samples collected from different rice growing locations and the information of rice blast isolates is present in Table 1.

The symptoms on the leaves collected from different cultivars of different places showed spindle-shaped lesions, with pointed ends and grey to white centers; reddish-brown to dark-brown margins (Fig. 1).

The colony colour was greyish or greyish white. Conidia were produced in clusters on long septate, slender conidiophores.

Conidia were hyaline, pyriform, usually 2 septate and 3 celled.

On the basis of colony characters and conidial nature the isolated pathogen was identified as *M. grisea*.

The characteristics of the pathogen were similar to the descriptions given by Ou (1985). These results were as similar that of Panda *et al.*, (2017) Sahu *et al.*, (2018).

Morphological characterization of blast isolates

The colony and morphological characteristics of the fungus are the important basic factors for identification of a fungus and identification of different form of a fungus. The morphological characteristics such as colony colour, colour of the mycelium, and surface appearance were studied among the isolates of *M. grisea* (Table 2; Fig. 2). The present study results are in concurrence with the findings of Srivastava *et al.*, (2014) who reported that the colony colour of the *M. grisea* varies from buff to black colour with smooth and rough colony margin. Hossain (2000) recorded that the mycelium of blast fungus in cultures was first hyaline in colour, then changed to olivaceous, 1–5.2 μm in width, septate and branched. The spore measurements were 15– 22 μm x 4–7 μm . Similarly, Meena (2005) reported that the colony colour of all the blast (*M. grisea*) isolates was usually buff with good growth on oat meal agar, greyish black with medium growth on host seed extract plus 2% sucrose agar, the raised mycelial growth with smooth colony margin on potato dextrose agar and raised mycelium with concentric ring pattern on Richard's agar medium.

Fig.1 Symptoms of blast disease



Fig 2: Morphology of ten blast isolates

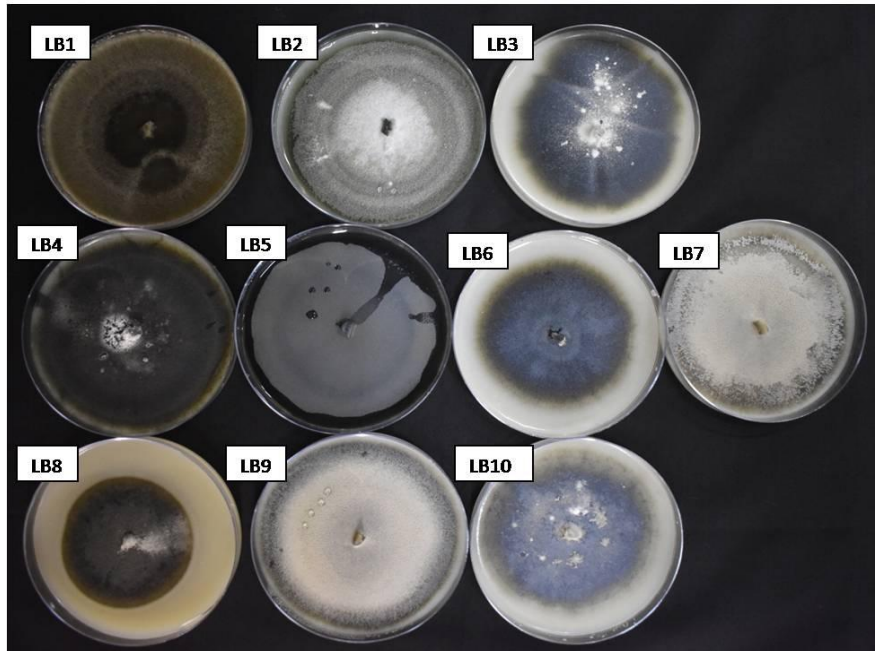


Fig 3: PCR amplification of ITS region

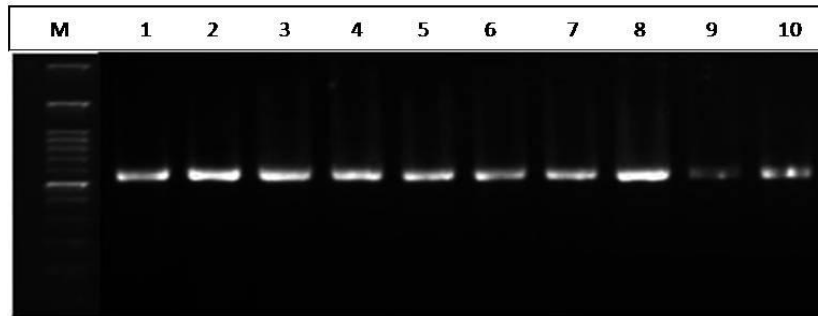


Fig.4 Antagonistic effect of *Bacillus* sp against *M. grisea*

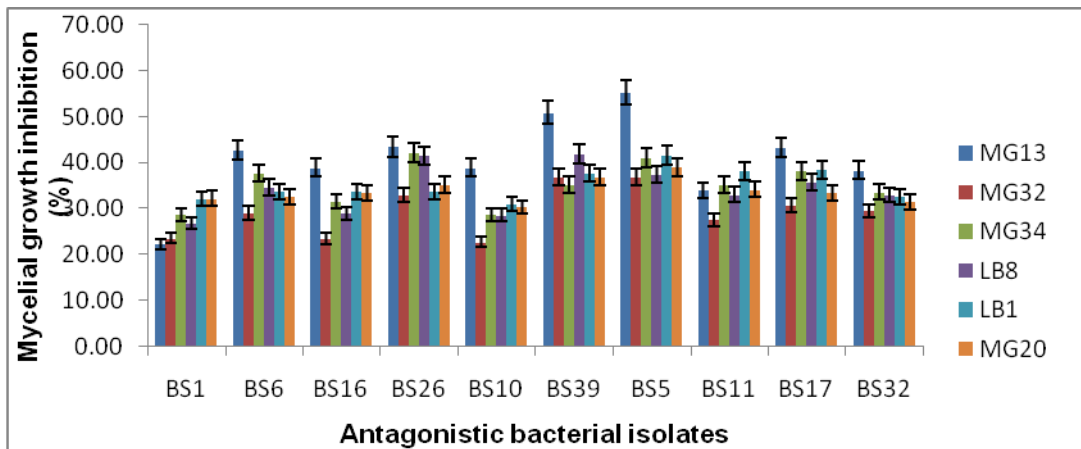


Fig.5 Antagonistic activity of Strain BS5 against *M. grisea* isolates

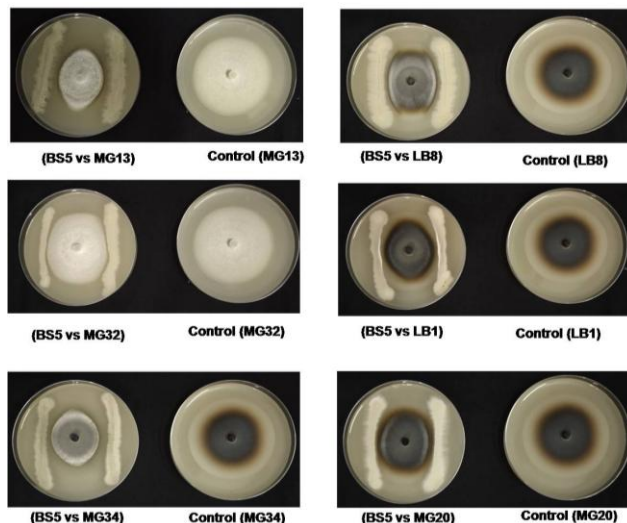


Table.1 Location of blast isolates used for the present study

S. No	Isolate name	Variety	Location
1	LB1	Sabita	ICAR-NRRI, Cuttack
2	LB2	Pooja	Kishor Nagar, Cuttack
3	LB3	Swarna	ICAR-NRRI, Cuttack
4	LB4	CR-1009	Patraput, Jeypore
5	LB5	Swarna	Kishor Nagar, Cuttack
6	LB6	Kalajeera	Pipil, Puri
7	LB7	Bhatagunda	Kishor Nagar, Cuttack
8	LB8	Mugudi	ICAR-NRRI, Cuttack
9	LB9	Lalat	Pipil, Puri
10	LB10	Kalajeera	ICAR-NRRI, Cuttack

Table.2 Morphological characterization of *M. grisea* isolates

S.N.	Name of the isolate	Surface appearance	Colony color and texture
1	LB1	Smooth	grey
2	LB-2	Smooth	white grey with cottony growth
3	LB-3	Rough	black
4	LB-4	Rough	black
5	LB-5	Smooth	grey
6	LB-6	Smooth	black
7	LB-7	Rough	white grey with cottony growth
8	LB-8	Smooth	black
9	LB-9	Smooth	white grey with cottony growth
10	LB-10	Rough	black

Molecular identification of *M. grisea*

The amplification of DNA sequences through the polymerase chain reaction (PCR) has found widespread application in the diagnosis and detection of fungi (Louis *et al.*, 2000). In our present study, PCR was performed to identify the *M. grisea* isolates using the universal primers of 18S (ITS 1) (5'-TCC GTA GGT GAA CCT GCG G-3') and 28S (ITS 4) (5'-TCC TCC GCT TAT TGA TAT GC-3'). The PCR reaction allowed amplifying the fungal ITS fragments of 550 bp (Fig. 3). All the forty eight isolates had the expected specific size of 550 bp which depicts molecular based confirmation of *M. grisea*. These results are correlated with the observations performed by other authors who found that ITS region varied from 750 to 1050 bp (Allain-Boule *et al.*, 2004; Levesque and De Cock, 2004).

Antagonistic effect of *Bacillus* sp against *M. grisea*

The antagonistic effect of *Bacillus* spp. were calculated as inhibition of mycelial growth by dual culture method and presented in Figure 6. Totally ten strains of *Bacillus* spp. were screened against six isolates of *M. grisea* to test their efficacy. Among the strains, BS5 showed maximum mycelial growth inhibition followed by BS26 (Fig. 4 and 5). Mycelial growth inhibition or antagonism might be due to the production of hydrolytic enzymes, antibiotics and volatile compounds (Prabhukarthikeyan *et al.*, 2014 b; 2016). Wulff *et al.*, (2002) reported the antagonistic activity of *B. subtilis* and *B. amyloliquefaciens* against black rot of cabbage *in vivo* and the metabolic profiles produced *viz.*, surfactin, iturin, bacillomycin and azalomycin F were responsible for the inhibition of *Xanthomonas campestris* pv. *campestris*. *Bacillus* species express antagonist activity by suppressing the

pathogen under *in vitro* and *in vivo* conditions (Elanchezhian *et al.*, 2018; Arrebola *et al.*, 2010). From the above evidences, it is assumed that production of antibiotics and secondary metabolites by endophytic *Bacillus* might have inhibited the growth of wilt pathogen under *in vitro* conditions. Secondary metabolites of PGPR showed an efficient antagonist effect against four phytopathogens tested in dual culture technique (Ramyasmruthi *et al.*, 2012).

In conclusion, the rice blast isolates were isolated, identified by morphological and molecular methods. Biocontrol is an alternative method for the chemical fungicides. Hence, the current study has provided a scope for better disease management in a sustainable manner.

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